EXHIBIT A

Product specification

FluoroLink™ Cy3 reactive dye 5-pack

PA 23000

Reagents for the labelling of biological compounds with Cy™3 bisfunctional dye

Safety warnings and precautions

Warning: For research only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. We recommend that this product and components are handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. As all chemicals should he considered as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves. Care should he taken to avoid contact with skin or eyes. In case of contact with skin or eyes, wash immediately with water.

CAUTION: This dye is intensely coloured and very reactive. Care should be exercised when handling the dye vial to avoid staining clothing, skin, and other items.

Storage

Store refrigerated at 2-8°C in the dark. Do not use if desiccant capsule in foil pack is not blue.

Introduction

Cyanine reagents have been shown to be useful as fluorescent labels for biological compounds[1,5]. These dyes are intensely fluorescent and highly water soluble, providing significant advantages over other existing fluorophores[4].

The Cy3 dye is an orange fluorescing cyanine that produces an intense signal easily detected using fluorescein filter sets. The Cy3 dye supplied here is a bisfunctional NHS-ester, and is provided in a dried, pre-measured form ready for the labelling of compounds containing free amino groups.

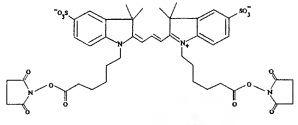

Figure 1. Cy3 bis functional dye

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Materials supplied

Five foil packs, each containing dried dye to label 1mg of protein
Product specification sheet with instructions for using the dye

Materials required but not supplied

Conjugation buffer: 0.1M sodium carbonate buffer (pH9.3)
Separation column containing a permeation gel (Sephadex™ G-50, or Bio-Gel™ P-10, minimum of 1cm diameter and 12cm length packed volume)
Separation buffer: phosphate-buffered saline, pH7.2, containing 0.1% sodium azide
Test tubes, transfer pipettes, glassware

Recommended procedure for use

This protocol has been designed for the preparation of Cy3-labelled IgG antibodies. It is designed to label 1mg protein to a final molar dye/protein (D/P) ratio between 4-12. This assumes an average protein molecular weight of 155000 daltons. Other D/P ratios can be obtained by using different amounts of protein.

NOTE: The following materials and procedures have been optimised for IgG antibodies. Other proteins may also be readily labelled, however, choice of buffers, separation media, and technique may vary in order to produce optimal results.

Altering the protein concentration and reaction pH will change the labelling efficiency of the reaction. Optimal labelling generally occurs at pH9.3. Proteins have been successfully labelled with this dye at a pH as low as 7.3, however, labelling times must be significantly longer at lower pH[1]. Higher protein concentrations usually increase labelling efficiency. Solutions of up to 10mg/ml protein have produced good conjugation reactions.

Conjugation of dye to antibody

Antibody to be conjugated should be dissolved at 1mg/ml in sodium carbonate-sodium bicarbonate buffer[2]. Add the protein solution (1ml) to the dye vial, cap the vial, and mix thoroughly. Care should be taken to prevent foaming of the protein solution. Incubate the reaction at room temperature for 30 minutes with additional mixing approximately every 10 minutes.

NOTE: Buffers containing primary amino groups such as TRIS and glycine will inhibit the conjugation reaction.
The presence of low concentrations (<2%) of biocides such as azide or thimerosal do not affect protein labelling.

Separation of protein from free dye

Labelled antibody can be separated from the excess, unconjugated dye hy gel permeation chromatography. It is convenient to pre-equilibrate the column with phosphate-buffered saline and to elute the protein using the same buffer. Two pink bands should develop during elution.

The faster moving band is Cy3-labelled antibody while the slower band is free dye. Many Cy3-labelled proteins can be stored at 2-8°C without further manipulation.

Labelled antibody can also be separated from unconjugated dye by dialysis. Dialysis does not give as efficient and rapid a separation as gel filtration. We therefore recommend that protein purification by gel filtration be used.

Estimation of final dye/protein (D/P) ratio

Dilute a portion of the labelled protein solution so that the maximum absorbance is 0.5 to 1.5AU. Molar concentrations of dye and protein are calculated, and the ratio of these values is the average number of dye molecules coupled to each protein molecule. Molar extinction coefficients of $150000M^{-1}cm^{-1}$ at 552nm for the Cy3 dye and $170000M^{-1}cm^{-1}$ at 280nm for the protein are used in this example. The extinction coefficient will vary for different proteins. The calculation is corrected for the absorbance of the dye at 280nm (approximately 8% of the absorbance at 552nm).

$[\text{Cy3 dye}]=(A_{552})/150000$

$[\text{antibody}]=[A_{280}-(0.08 \bullet A_{552})] / 170000$

$(D/P)_{final}=[\text{dye}]/[\text{antibody}]$

$(D/P)_{final}=[1.13 \bullet (A_{552})] / [A_{280}-(0.08 \bullet A_{552})]$

Conjugation of dye to oligonucleotides

Modified oligonucleotides containing alkyl amino groups can be labelled with cyanine dye. Synthetic oligonucleotides must be deprotected before conjugation. Procedures that use concentrated ammonium hydroxide require the following pretreatment to remove all traces of ammonia.

Concentrate the sample until it is dry (a vacuum concentrator works effectively).

Dissolve the sample in 0.25ml of a 0.5M sodium chloride solution and separate using an appropriate desalting column (Bio-Gel™ P-4 or equivalent) equilibrated with a 5.0mM borate buffer solution adjusted to a pH of 8.0. Elute the sample with above borate buffer solution.

Concentrate the sample until it is dry. Dissolve the dry sample in a 0.1M carbonate buffer (pH 8.5-9.0).

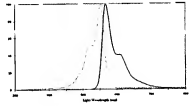
Conjugation is carried out by adding 30nmoles of oligonucleotide sample is approximately 0.5ml of carbonate buffer to the dye vial. Cap the vial and mix thoroughly. Incubate the reaction at room temperature for 60 minutes with additional mixing at 15 minute intervals.

Separation of labelled oligonucleotides

Conjugated oligonucleotides can be separated from free dye using the same gel filtration procedures listed for separating conjugated antibody. A gel with a smaller exclusion size (such as Bio-Gel P-4) and a longer column length must be used with shorter oligonucleotides in order to ensure complete separation.

Cy3-labelled oligonucleotides can be separated from unconjugated oligonucleotides using RP-HPLC. The general procedure listed in reference 3 may be optimised for the specific nucleotide sequence and HPLC configuration.

Figure 2. Cy3 dye absorption and fluorescence spectra



Cy3 bisfunctional dye characteristics

Formula weight	.....	949.11
Absorbance max	.....	550nm
Extinction max	.....	150000 $M^{-1}cm^{-1}$
Emission max	.....	570nm
Quantum yield	.....	>0.15*

* for labelled proteins, D/P=2

References

1. MUJUMDAR, R.B. *et al., Bioconjugate Chemistry* 4 (2), pp.105-111, 1993.
2. SOUTHWICK, P.L. *et al., Cytometry*, 11, pp.418-430, 1990.
3. SMITH, L.M. *et al., Nucleic Acids Research*, 13, pp.2399-2412, 1985.
4. WESSENDORF, M.W. and BRELJE, T.C., *Histochemistry*, 98 (2), pp.81-85, 1992.
5. YU, H. *et al., Nucleic Acids Research*, 22 (15), pp.3226-3232, 1994.

http://www.gehealthcare.com/lifesciences
GE Healthcare UK Ltd GE Healthcare Place
Little Chalfont Buckinghamshire England HP7 9NA
GE Healthcare Bio-Sciences AB SE-751 84 Uppsala Sweden
GE Healthcare Bio-Sciences Inc 800 Centennial Avenue PO Box 1327 Piscataway NJ08855 USA

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